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The Effect of Resveratrol Supplementation on Serum Levels of Asymmetric De-Methyl-Arginine and Paraoxonase 1 Activity in Patients with Type 2 Diabetes: A Randomized, Double-Blind Controlled Trial

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29 **ABSTRACT**

30 **Objective:** The present study sought to investigate the effect of micronized resveratrol
31 supplementation on serum levels of asymmetric de-methyl-arginine (ADMA) and paraoxonase-1
32 (PON1) activity in patients with type 2 diabetes (T2D).

33 **Methods:** In this double-blinded randomized trial, 76 patients with T2D were recruited.
34 Participants were randomly assigned to consume 1000 mg resveratrol or placebo capsules
35 (methylcellulose) per day, for 8 weeks. Serum levels of ADMA and PON1 enzyme activity were
36 measured at the beginning and end of the intervention using the ELISA method. In total, 71
37 participants completed the study.

38 **Results:** Our results showed that resveratrol significantly decreased serum levels of ADMA (-
39 0.16 ± 0.11 , $P < 0.001$) and improved PON1 enzyme activity (15.39 ± 13.99 , $P < 0.001$) compared with
40 placebo, after adjusting for confounding factors (age, sex and baseline body mass index).

41 **Conclusion:** Our findings suggest that 8-week resveratrol supplementation may produce
42 beneficial effects on serum levels of ADMA and PON1 enzyme activity in patients with T2DM.
43 However, further research is needed to confirm the veracity of these results.

44 **Keywords:** *Resveratrol, ADMA, PON1 protein, Type 2 diabetes mellitus*

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INTRODUCTION

Type 2 diabetes (T2D), the most prevalent endocrine disease, represents one of the most important health issues affecting people globally (1, 2). Empirical evidence indicates that cardiovascular disease is a major cause of mortality and morbidity in patients with diabetes (3). Obesity, dysglycemia, dyslipidemia, and hypertension represent the most important risk factors for cardiovascular diseases, which are especially common in diabetic patients (4). The vascular endothelium plays a pivotal role in maintaining the vascular tone and mediates production (5). One of the mediators is nitric oxide (NO), which is produced in response to stress, and has an important function in vasodilatation and increases circulation (6). Asymmetric dimethylarginine (ADMA) is a competitive endogenous inhibitor for nitric oxide synthase (NOS) and inhibits the production of NO in pathological concentrations (7). Increased serum levels of ADMA have been reported in patients with diabetes, renal failure, hypercholesterolemia, cardiovascular diseases, and hypertension (8).

Chronic hyperglycemia in T2D induces oxidative stress in various pathways, such as; glucose auto-oxidation, glycosylation of operational proteins, activation of the polyol pathway, endothelial NOS (eNOS) uncoupling and oxidative phosphorylation (9-12). Paraoxonases (aryl dialkyl phosphatase) as antioxidant factors, also initially identified as hydrolyzing enzymes of organophosphorus compounds such as, paraoxon, or diazoxone insecticides (13, 14). Paraoxonase-1 (PON1) is an esterase which is produced in the liver and is transported with circulating high-density lipoprotein (HDL) (15, 16). It seems that PON1 is partly responsible for the antioxidant property of HDL (17). Some studies have shown that the PON1 activity is independent of the amount of Apo-lipoprotein HDL (18); PON1 also inhibits LDL peroxidation and oxidized LDL synthesis (19), and hydrolyzes homo-cysteine, which is an important risk factor for cardiovascular

disease (20). PON1 activity is important in the prevention of atherosclerosis progression by inhibition of MCP-1 production (Monocyte Chemoattractant Peptide 1), which is stimulated by oxidized LDL in the endothelial cells (21). Some previous studies have reported that PON1 enzyme activity may be decreased in diabetic patients (22-24), whilst high serum levels of glucose can lead to PON1 separation from HDL (25). Furthermore, it seems that serum levels of ADMA and PON1 activity are affected by antioxidants (26).

Resveratrol is a polyphenol found mostly in grapes and nuts and has been shown to elicit beneficial effects on diabetes and cardiovascular diseases (27, 28). The cardiovascular protective effects of resveratrol have been widely investigated; however, the exact mechanisms are far from consensual. The results of some meta-analytical studies have shown that resveratrol supplementation can elicit improvements in endothelial function (29), and reductions in inflammatory markers (30-32); however, a previous meta-analysis concluded that resveratrol supplementation has no significant effects on cardiovascular risk factors (33). In the present study, we investigated the effects of resveratrol supplementation on serum levels of ADMA and PON1 activity in patients with type 2 diabetes.

MATERIALS AND METHODS

Study Design and Participants

Patients with T2D were selected from a diabetes center (Yazd, Iran), and the diagnosis of diabetes was confirmed by an endocrinologist (34). The protocol of the present double-blind randomized controlled trial was approved by the Ethics Committee of Shahid Sadoughi University of Medical Sciences in Yazd (IR.SSU.SPH.REC.1397.073) and registered in the

Iranian Registry of Clinical Trials (www.irct.ir) as IRCT20171118037528N1). Informed consent was provided by all participants prior to study commencement.

Inclusion and Exclusion Criteria

Detailed information about the study design has been previously described in detail (35). Briefly, men and women with T2D aged 30-60 years old, body mass index (BMI) of 25-30 kg/m², and glycated hemoglobin (HbA1c) lower than 8% were enrolled in the study. Exclusion criteria included; diagnosed kidney or liver disease, cancer, Alzheimer's, gastrointestinal ulcer, inflammatory and autoimmune diseases, and/or history of myocardial infarction, treatment with any supplement containing antioxidants, insulin, fibrates, warfarin, aspirin or any drugs that inhibit platelet aggregation in the 6 months preceding the study. Patients who consumed alcoholic beverages habitually, and pregnant or lactating women were also excluded.

Setting

A stratified randomized method, using a computer random generated number based on sex and age (30-45, 45-60 years old), was used to assign participants into the intervention or control group, respectively. Patients in the intervention group received two capsules per day, which provided 1000 mg/day purified resveratrol (Mega-Resveratrol, Danbury, USA) for 8 weeks. Two capsules containing methyl cellulose (Barij essence, Kashan, Iran) were taken by patients in the control group for the same duration. The placebo was similar in appearance and taste with the resveratrol supplement. Patients were not deprived of their usual treatment for diabetes.

A person outside the research team performed the packing and labeling (A or B) of the bottles containing resveratrol and placebo. The researchers and participants were not aware of the contents until the end of the intervention. Patients were asked to report any suspected adverse events. The compliance rate of the participants was evaluated using the remaining capsule counts at the end of

the study, and participants were asked to maintain their habitual diet and physical activity throughout the study.

Nutritional and Physical Activity Assessment

To assess nutrient intake, two, 3-day dietary food records (one weekend day and two weekdays) were completed by the participants in the first and last week of the intervention. Data were analyzed using Nutritionist IV software (The Hearst Corporation, San Bruno, California, USA).

To assess the physical activity level, metabolic equivalent (MET) was calculated using a validated questionnaire at the beginning and end of the study (36). In this questionnaire, information on physical activity is classified based on the intensity of each activity in nine different categories (ranging from inactivity to severe sports activities). The duration of each activity was multiplied by the coefficient for each activity, and the values obtained in the nine different classes were summed in order to provide MET/h per day.

Anthropometric and Biochemical Measurements

Anthropometric measures, including height, body weight, waist and hip circumferences, BMI, fat and, fat-free masses, were assessed before and after the intervention using a segmental body composition analyzer (Tanita BC-418, Tokyo, Japan). The results of the anthropometric measures, as well as cardio-metabolic biochemical factors (glycemic indices and lipid profile), have been reported elsewhere (37).

Blood samples for biochemical parameters were collected at the beginning and end of the study after 12h nocturnal fasting. Blood samples were centrifuged for 10 minutes at room temperature (3000 g; Eppendorf AG, Hamburg), and then the serum samples were frozen at -70 ° C until analyses. Serum levels of ADMA were measured applying enzyme-linked immunosorbent assay (ELISA) method using a commercially available kit (Zellbio, Germany) with inter-and intra-

assay<12% and <10%, respectively. The PON1 activity also determined by the ELISA method using a commercially available kit (Zellbio, Germany, inter-and intra-assay: CVs were 4.8% and 4.1%, respectively).

Sample Size and Statistical Analysis

This report is part of a previous study that calculated the sample size based on the *PPARα* gene expression in peripheral blood mononuclear cells (35). Although, a retrospective power analysis was performed to assess the quantity of the sample size for our interested outcomes. The results showed adequate power for ADMA levels (observed power= 1.0).

SPSS software for windows version 23.0 (SPSS, Chicago, IL, USA) was used for all data entry and statistical analyses. The values were expressed as mean ± standard deviation for continuous and proportions for categorical data. The Kolmogorov-Smirnov test was used to evaluate the distribution of variables. To compare the quantitative values between the two groups, an independent samples t-test and within groups paired t-test were used, respectively. Analysis of covariance (ANCOVA) was used to modify possible confounding factors including age, gender, and baseline BMI. Statistical significance was accepted, *a priori*, at $P < 0.05$.

RESULTS

Of the 76 participants enrolled in the study, five patients did not complete the intervention due to pregnancy (n=1), traveling (n=1) and withdrawal of consent (n=3). Finally, data from 71 participants (35 patients in resveratrol and 36 patients in placebo groups) were included in the analysis (**Figure 1**). More than 90% compliance (92.6% in placebo and 93.1% in resveratrol) was detected through capsule counting, and no adverse side effects were reported.

Table 1 details the general characteristics of the participants before the intervention, and there were no significant differences in baseline variables between the two groups. The mean age of

participants in resveratrol and placebo groups was 50.14 ± 7.38 and 50.06 ± 7.69 years, respectively. No significant between-group differences for dietary intake and physical activity were observed at the baseline and they also did not change following the 8-week intervention (**Table 2**).

Resveratrol significantly reduced ADMA levels compared with baseline and the placebo group (-0.16 ± 0.11 (ng/ml); all P-values < 0.001). PON1 activity was also significantly increased after supplementation in the resveratrol group (15.39 ± 13.99 (U/L); $P < 0.001$) and compared with the placebo group ($P = 0.04$). These findings remained significant after adjusting for confounding variables (all P-values < 0.001) (**Table 3**).

DISCUSSION

The results of the current study showed a significant reduction in serum levels of ADMA, and significant increase in PON1 activity, following 8-week resveratrol supplementation. In line with our findings, previous studies have reported a significant increase in PON1 activity after resveratrol (38), pomegranate juice (39), eicosapentaenoic acid (40), barberry juice (41), and vitamin E supplementation (42) in patients with type 2 diabetes. Furthermore, one study reported a higher intake of fruit and vegetable leads to an increase in PON1 activity (43). The findings of some in-vitro studies have also showed that resveratrol increases PON1 gene expression and activity in different human cells (44-46).

PON1 is a HDL-associated enzyme that hydrolyzes oxidized LDL-cholesterol, and is known for its atheroprotective capabilities (47). Furthermore, this enzyme plays a critical role in the protection against oxidative stress-related diseases (48, 49); including cardiovascular diseases, the

183 major cause of mortality among patients with diabetes (50). Moreover, the activity and
184 concentration of PON1 are reported to decrease in these patients (23).

185 Resveratrol is an antioxidant that appears to affect PON1 activity through several pathways.
186 Resveratrol can result in an increase in carnitine palmitoyl transferase-1, decrease in acetyl-CoA
187 carboxylase and fatty acid synthase genes expression, and, consequently, an elevation in HDL
188 levels (38). The results of the present study also support the beneficial effect of resveratrol on HDL
189 levels (37). Furthermore, it seems that resveratrol might regulate gene expression by binding to
190 the estrogen response element-2 sequences (51). There are similar sequences in the promoter
191 region of PON1 gene, suggesting that PON1 gene expression upregulation induced by resveratrol
192 may be related to the presented sequences (52). Moreover, resveratrol is known as a ligand for
193 aryl-hydro carbon receptors (AhR) and can increase PON1 gene expression and activity through
194 AhR-dependent mechanisms (53).

195 In the present study, we also observed a significant reduction in serum levels of ADMA following
196 resveratrol supplementation. Previous reports have identified that increased ADMA levels are
197 associated with oxidative stress related diseases, such as diabetes (54-56). ADMA is produced via
198 protein arginine methyl transferase and breaks down to citrulline and dimethyl amine by dimethyl
199 arginine dimethyl amino hydrolase (DDAH). Oxidative stress reduces the gene expression and
200 activity of DDAH resulting in endothelial dysfunction (57, 58), whilst there is substantive evidence
201 that increased ADMA levels contribute to injuries induced by oxidative stress (59). There are
202 numerous reports in the literature asserting that resveratrol can stimulate eNO synthesis and inhibit
203 its degradation in several mechanisms (60-62). However, one study suggested that the levels of
204 eNOS did not significantly change following resveratrol supplementation (63); conceivably due to
205 the small sample size (n=48). Resveratrol activates sirtuin-1 (SIRT1) through AMP-activated

protein kinase (AMPK) pathway (64), and SIRT-1 increases eNOS gene expression by deacetylating Forkhead box O (FOXO) transcription factors (65). It has been shown that elevated NO levels can upregulate DDAH by cyclic GMP induction and subsequently decreased ADMA levels (66). There is also some evidence that resveratrol can independently upregulate DDAH gene expression (67); however, the molecular mechanisms are not well identified. Moreover, DDAH upregulation causes decreases ADMA levels and increases NO production and bioavailability (58, 68).

A number of in-vitro studies in endothelial cells have reported significant decreases in ADMA levels after red wine consumption as a source of resveratrol (58). Some RCTs have also shown that ADMA levels are reduced after coenzyme Q10 (69), alpha-lipoic acid (70, 71), eicosapentaenoic acid (72), and DHA-enriched fish oil consumption (73) in patients with type 2 diabetes, respectively, and also vitamin E supplementation in chronic kidney disease patients (74). Moreover, one animal study indicated DDAH activity increased after intervention with *trans*-3, 5, 4'-trihydroxystilbene as an analog of resveratrol on gastric mucosal injury (67). However, the results of some studies are inconsistent with our results. For instance, one study indicated that vitamin C and E did not affect ADMA levels in children with hyperlipidemia (75), whilst another study reported no significant differences in PON1 activity when omega-3 was administered (76). However, the small number of participants may justify the aforementioned findings.

To the authors' knowledge, this is the first clinical trial study to investigate the effect of resveratrol supplementation on serum levels of ADMA. Although there is one RCT investigating the effect of resveratrol on PON1 activity in patients with diabetes (38), we utilized micronized resveratrol to increase bioavailability, and included patients with overweight exclusively, to adjust oxidative stress induced by obesity. Stratification by gender and age also permitted us to control confounders

related to these factors. Despite the novelty of the present study, there are some limitations that must be considered. The present study was designed for short-term assessment of resveratrol supplementation effects; thus, we have no information as to the longer-term effects, or dose-response relationship beyond this time. Finally, we did not investigate the cellular pathways related to the beneficial effects of resveratrol on our interested outcomes; which clearly represents an avenue for future research.

Conclusion

The findings of the present study demonstrated that 8-week resveratrol supplementation can significantly improve ADMA levels and enhance PON1 activity in patients with diabetes. These findings may support the beneficial, atheroprotective, effects of resveratrol; although, more research is needed to confirm the veracity of our findings.

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Conflict of interest

There is no conflict of interest in the present study.

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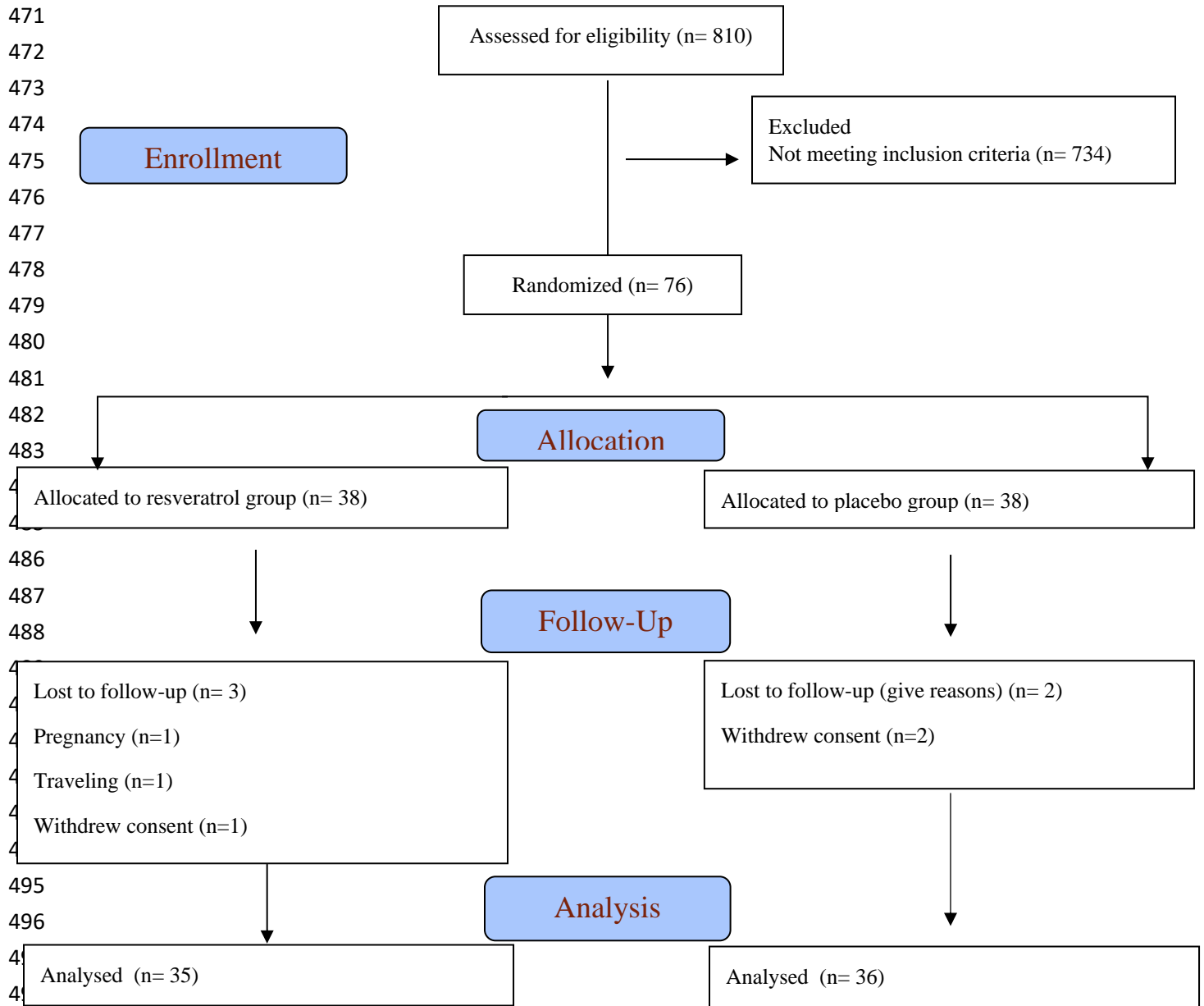


Figure 1. CONSORT diagram outlining the number of subjects involved in enrollment, intervention allocation, follow-up, and data analysis.

507 **Table 1.** Baseline characteristics of the study participants¹

Variable	Resveratrol (n=35)	Placebo (n= 36)	P-value ²
Age (years)	50.14 ± 7.38	50.06± 7.69	0.96
Diabetes duration (years)	9.40 ± 7.07	8.11 ± 6.90	0.44
Gender (female), n (%)	15 (42.9)	16 (44.4)	0.89
Menopause status, n (%)	4 (26.6)	3 (18.8)	0.68
Smoker, n (%)	5 (14.3)	2 (5.6)	0.21
HbA1C (%)	7.33± 0.65	7.33± 0.65	0.92
Complications			
Hypertension, n (%)	11 (31.4)	7 (19.4)	0.24
Kidney stone, n (%)	2 (5.7)	3 (8.3)	0.66
Non-alcoholic fatty liver, n (%)	3 (8.6)	2 (5.6)	0.62
Neuropathy, n (%)	2 (5.7)	2 (5.6)	0.97
Retinopathy, n (%)	5 (14.3)	5 (13.9)	0.96
Family T2DM History, n (%)	25 (71.4)	30 (83.3)	0.23
Medications			
Metformin, n (%)	30 (85.7)	31 (86.1)	0.96
Glibenclamide, n (%)	11 (31.4)	16 (44.4)	0.25
Statins, n (%)	3 (8.6)	4 (11.1)	0.70
Blood pressure lowering drugs, n (%)	6 (17.1)	5 (13.9)	0.72
Anthropometric measures			
Weight (kg)	73.69± 8.24	72.71± 10.52	0.66
Height (cm)	164.94 ± 7.22	162.08 ± 11.29	0.20
BMI (kg m ⁻²)	27.10± 2.69	27.66± 2.71	0.39
HC (cm)	101.97± 6.05	103.47± 8.04	0.37
WC (cm)	91.75± 7.4	92.58± 8.53	0.66
WHR	0.9± 0.06	0.89± 0.05	0.53
WHtR	0.55± 0.05	0.57± 0.07	0.25

¹Data are expressed as mean ± SD for continuous variables or as frequency and percentage for categorical variables.

²Differences between the control and intervention groups were evaluated using the Independent sample t-test for continuous variables and chi-square test for categorical variables. BMI, Body mass index; HbA1c, glycated hemoglobin; HC, Hip circumference; WC, Waist circumference; WHR, Waist to hip ratio; WHtR, Waist to height ratio

Table 2. Dietary intake and physical activity during study in resveratrol and placebo groups (mean \pm SD)

¹The presented P-values are associated with within-group comparisons obtained paired t test.

Variable	Resveratrol (n=35)			Placebo (n= 36)			
	Before	After	P-value ¹	Before	After	P-value ¹	P-value ²
Energy (kcal)	1612.87 \pm 587.87	1544.71 \pm 597.37	0.45	1708.79 \pm 515.39	1674.16 \pm 597.07	0.55	0.47
Carbohydrate (%)	59.76 \pm 12.71	61.36 \pm 11.2	0.43	60.82 \pm 9.96	60.61 \pm 8.76	0.88	0.7
Protein (%)	15.5 \pm 4.65	16.28 \pm 5.17	0.47	15.48 \pm 3.48	15.84 \pm 4.02	0.56	0.97
Fat (%)	25.34 \pm 14.55	24.14 \pm 11.02	0.58	24.61 \pm 10.42	24.26 \pm 9.63	0.77	0.81
Fiber (g/d)	9.43 \pm 4.11	9.69 \pm 4.32	0.81	10.44 \pm 5.23	10.86 \pm 5.27	0.64	0.2
Cholesterol (mg/d)	219 \pm 29	208 \pm 47	0.77	189 \pm 71	191 \pm 63	0.61	0.75
PUFA (%)	8.22 \pm 4.13	8.28 \pm 4.24	0.81	9.13 \pm 4.35	9.71 \pm 5.12	0.43	0.76
MUFA (%)EPA	6.32 \pm 4.21	6.12 \pm 5.1	0.53	5.67 \pm 3.72	5.82 \pm 3.22	0.62	0.41
(%)	0.01 \pm 0.69	96.62 \pm 571.66	0.32	0.0038 \pm 0.0097	0.0007 \pm 0.0014	0.14	0.17
Zinc	6.72 \pm 2.64	6.79 \pm 3.12	0.64	7.03 \pm 2.83	7.73 \pm 4.004	0.71	0.65
Vitamin E	3.45 \pm 2.01	4.44 \pm 5.44	0.25	3.75 \pm 2.96	4.22 \pm 3.82	0.28	0.64
Vitamin C	57.24 \pm 49.18	53.09 \pm 54.36	0.73	64.36 \pm 44.64	51.31 \pm 41.69	0.23	0.55
Selenium	0.09 \pm 0.47	0.10 \pm 0.06	0.15	0.08 \pm 0.07	0.109 \pm 0.1005	0.06	0.66
Beta-Carotene	365.67 \pm 816.21	206.60 \pm 424.54	0.25	289.63 \pm 694 \pm 62	348.61 \pm 640.04	0.45	0.69
DHA	0.05 \pm 0.18	19.20 \pm 111.93	0.32	0.005 \pm 0.01	0.007 \pm 0.01	0.21	0.12
PA (MET-h/d)	35.61 \pm 5.22	36.33 \pm 5.7	0.14	37.54 \pm 7.82	36.99 \pm 5.87	0.31	0.24

²The presented P-values are associated with baseline comparisons of the resveratrol and control groups obtained independent sample t test

EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid; MUFA, Mono-unsaturated fatty acid; PA, physical activity.

PUFA, Poly-unsaturated fatty acid.

Table3: Comparison of serum levels of ADMA and PON1 enzyme activity at baseline and after intervention in resveratrol and placebo groups (mean \pm SD).

Variable	Resveratrol (n=35)				Placebo (n= 36)							
	Before	After	P-value ¹	Change	Before	After	P-value ¹	Change	P-value ²	P-value ³	P-value ⁴	P-value ⁵
ADMA (ng/ml)	0.61 \pm 0.47	0.44 \pm 0.38	0.000	-0.16 \pm 0.11	0.60 \pm 0.45	0.57 \pm 0.26	0.06	0.04 \pm 0.07	0.527	0.000	0.000	0.000
PON1 (U/L)	97.32 \pm 18.68	112.72 \pm 24.91	0.000	15.39 \pm 13.99	100.12 \pm 24.60	101.06 \pm 24.14	0.223	0.94 \pm 4.95	0.592	0.049	0.000	0.000

¹The presented P-values are associated with within-group comparisons obtained paired t test.

²The presented P-values are associated with baseline comparisons of the resveratrol and control groups obtained independent sample t test

³The presented P-values are associated with between groups comparisons after intervention obtained independent sample t test.

⁴ The presented P-values are associated with mean changes comparisons obtained from independent-sample t test.

⁵ The presented P-values are associated with mean changes comparisons adjusted for age, gender, and BMI obtained from analysis of covariance (ANCOVA).

ADMA, asymmetric de-methyl-arginine; PON1, paraoxonase 1.